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TRIMETHOPRIM-SULFAMETHOXAZOLE RESISTANCE IN *STAPHYLOCOCCUS AUREUS* IN AFRICA: DISTRIBUTION OF RESISTANCE GENES AND EVALUATION OF THE SUCCESS OF MAJOR MRSA CLONES

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ABSTRACT

Staphylococcus aureus is a major pathogen worldwide due to its remarkable ability to develop resistance to antibiotics coupled with the emergence of highly virulent strains. Trimethoprim-sulfamethoxazole (SXT) is a fixed-dose combination of the antifolate compounds trimethoprim and sulfamethoxazole, which act synergistically by inhibiting distinct steps in the synthesis of bacterial folic acid. SXT is recommended as a first-line treatment of uncomplicated urinary tract infections and skin and soft tissue infections. It is also prescribed for respiratory infections and is widely used in resource-constrained areas as a prophylaxy among HIV-infected or HIV-exposed children. Very high SXT resistance rates have been reported among *S. aureus* isolates from the African continent, namely among major methicillin-resistant *S. aureus* (MRSA) clonal types circulating in Portuguese-speaking African countries, such as Angola, São Tomé and Príncipe and Cape Verde.

The aim of the present study was to provide new insights on the high rates of SXT-resistant *S. aureus* isolates in the African continent. Furthermore, we also evaluated the success of three major MRSA clones in Portuguese-speaking African countries by comparing with the Brazilian MRSA-ST239-III (or variant ST241-III), also resistant to SXT, which is widely spread all over the word but not detected so far in Portuguese-speaking African countries.

Our results demonstrated for the first time an SXT-hetero-resistance phenotype, highly frequent in *S. aureus* isolates from Africa. We also evidenced a wide prevalence of *dfrG* gene in Portuguese-speaking African countries, reinforcing its potential origin in the African continent. Since these countries present important demographic and economic exchanges with Portugal, future spread of *dfrG* within MRSA in populations where antifolate resistance is currently considered to be low should be monitored. Moreover, we showed that the epidemiological success of the major MRSA clones in Portuguese-speaking African countries may be due to their capacity to survive under different stress situations including a better adaptation to alkaline conditions, and to their potential to outgrow other epidemic SXT-resistant MRSA lineages such as the Brazilian clone.

Keywords: *Staphylococcus aureus*, MRSA, antibiotic resistance, sulfonamides, trimethoprim, trimethoprim-sulfamethoxazole, Africa

RESUMO

Staphylococcus aureus é uma bactéria gram-positiva, imóvel, anaeróbia facultativa, pertencente à família Micrococcaceae. É um agente patogénico de maior relevância e mundialmente disseminado devido à sua notável capacidade em desenvolver resistência aos antibióticos, juntamente com a emergência de estirpes altamente virulentas. A bactéria tem adquirido sucessivamente resistência aos antimicrobianos introduzidos na prática clínica, nomeadamente aos β-lactâmicos, levando à emergência de isolados resistentes à meticilina (MRSA – do inglês, "methicillin-resistant *Staphylococcus aureus*"). Além da resistência a este β-lactâmico, os isolados de *S. aureus* podem igualmente adquirir resistência a múltiplas outras classes de antibióticos, reduzindo as opções de tratamento no caso de infeções causadas por esta bactéria. A bactéria é responsável por uma grande variedade de infeções de gravidade variável, desde infeções de pele menos graves a infeções sistémicas e fatais.

A propagação mundial de isolados MRSA deve-se sobretudo à disseminação de determinadas linhagens clonais epidémicas. Os clones MRSA são geralmente definidos pela combinação do perfil de MLST (do inglês "multilocus sequence type") e da cassete SCC*mec* (do inglês, "staphylococcal chromossome cassette *mec*") (i.e. ST239-III). Os principais clones MRSA associados ao ambiente hospitalar (HA-MRSA – do inglês "hospital-associated MRSA), incluem o clone Brasileiro (ST239-III), Nova Iorque/Japão (ST5-II), Pediátrico (ST5-VI e ST5-IV_a), EMRSA-15 (ST22-IV_h) e EMRSA-16 (ST36-II). Em relação às principais linhagens clonais MRSA associadas à comunidade (CA-MRSA – do inglês, "community-associated MRSA"), destacam-se os clones USA300 (ST8-IV_a), Europeu (ST80-IV_{NT} e ST80-IV_c), Sudoeste/Pacífico (ST30-IV), Queensland (ST93-IV) e ST59-IV.

No continente Africano verifica-se uma predominância de isolados MRSA associados ao complexo clonal (CC) 5, tais como o clone Pediátrico (ST5-IV_a), nomeadamente nos Países Africanos de Língua Oficial Portuguesa (PALOP). Além disso, vários clones CA-MRSA, incluindo o clone "West Australia MRSA-2" (ST88-IV_a) e o clone ST8-IV_a, são também muito frequentes, tanto no ambiente hospitalar como na comunidade. Os factores que contribuem para o sucesso destes tipos clonais MRSA no continente Africano são ainda desconhecidos.

O trimetoprim-sulfametoxazol (SXT) é uma combinação, de dose fixa, dos compostos antifolatos trimetoprim e sulfametoxazol, que atuam sinergicamente ao inibir várias etapas da síntese do ácido fólico bacteriano. O SXT está recomendado como primeira linha de tratamento de infeções não complicadas do trato urinário, e infeções da pele e tecidos moles. Este composto de amplo espectro é igualmente utilizado para o tratamento de infeções respiratórias, bem como para a profilaxia de pacientes infetados ou expostos ao Vírus da Imunodeficiência Humana (VIH). Tendo em conta a sua ampla utilização, e uma vez que o SXT é um antimicrobiano de baixo custo, bem tolerado, e com boa absorção pelos tecidos celulares, tem-se verificado um aumento significativo da prescrição médica deste antibiótico nos últimos anos, nomeadamente nos países em desenvolvimento.

A resistência ao sulfametoxazol em isolados de *S. aureus* é geralmente mediada por mutações não-sinónimas no gene *folP*, que codifica a proteína dihidropteroato sintetase (DHPS). Em relação ao trimetoprim, são conhecidos dois mecanismos de resistência: (i) a aquisição de genes de resistência (*dfrA*, *dfrG* and *dfrK*) transportados em plasmídeos, que codificam variantes da proteína DHFR e conferem uma resistência elevada ao antibiótico (CMI $\geq$ 512 μg/mL); e (ii) mutações não sinónimas no gene cromossómico *dfrB* que codifica a proteína intrínseca *S. aureus* DHFR (SaDHFR), conferindo uma resistência intermédia ao trimetoprim (CMI $\leq$ 256 μg/mL). Estudos quanto à contribuição relativa dos diferentes marcadores de resistência ao trimetoprim em isolados clínicos de *S. aureus* são escassos, e representam implicações de maior importância para o desenvolvimento de novos antimicrobianos antifolatos.

As taxas de resistência ao SXT variam consideravelmente consoante a zona geográfica e o período de isolamento dos isolados *S. aureus*. Embora a resistência a este antimicrobiano em isolados clínicos de *S. aureus* não seja muito comum na Europa, elevadas taxas de resistência têm sido descritas nos PALOP, nomeadamente em Angola, São Tomé e Príncipe e Cabo Verde, provavelmente devido ao uso frequente do antibiótico. Por outro lado, o clone Brasileiro (MRSA-ST239-III) ou variante (ST241-III), especialmente disseminado hoje em dia na Ásia e América do Sul mas nunca detetado nos PALOP, tem sido normalmente associado à resistência ao SXT.

O presente estudo teve como principal objetivo fornecer informação quanto às elevadas taxas de resistência ao SXT em isolados de *S. aureus* do continente Africano, (i) determinando pela primeira vez a prevalência dos diferentes marcadores de resistência ao trimetoprim em isolados recolhidos nos PALOP, nomeadamente em São Tomé e Príncipe, Angola e Cabo Verde e (ii) avaliando o "fitness" e a resistência a diferentes condições de "stress" químico em representativos dos três principais clones MRSA nos PALOP – ST5-IV_a, ST88-IV_a e ST8-IV_a – todos resistentes ao SXT. Por outro lado, pretendemos perceber quais os fatores que contribuem para o sucesso dos principais clones MRSA disseminados nos PALOP e comparar com o clone MRSA Brasileiro (ST239-III) ou variante (ST241-III), igualmente resistentes ao SXT.

Os nossos resultados demonstraram pela primeira vez um fenótipo de hetero-resistência para o SXT em isolados de *S. aureus*, fenómeno este muito frequente em isolados de *S. aureus* recolhidos nos países Africanos e que por conseguinte necessita de estudos adicionais. Foi evidenciada uma elevada prevalência (81,1%) do gene *dfrG* nos PALOP, reforçando a sua possível origem no continente Africano. Cerca de 20% dos isolados apresentaram o gene *dfrA*, maioritariamente associado a isolados MRSA pertencentes ao clone ST88-IV_a. No entanto o gene *dfrK* não foi detetado em nenhum isolado. Uma vez que o gene *dfrG* é facilmente transferido através de elementos genéticos móveis e que Portugal detém importantes trocas demográficas e económicas com os PALOP, a eventual propagação do gene *dfrG* em isolados MRSA em populações onde a resistência aos antifolatos é atualmente considerada ainda baixa deve ser monitorizado. Sequenciámos ainda o gene *dfrB* em 10 dos 15 isolados resistentes ao trimetoprim pertencentes ao clone Brasileiro e que não apresentaram *dfrG*, *dfrA* nem *dfrK*, verificando-se três mutações sinónimas e duas não sinónimas (F99Y e R150H) em todos os isolados. Os restantes cinco isolados, do Brasil (n = 2), Argentina (n = 1), Taiwan (n = 1) e Portugal (n = 1), não amplificaram nenhum dos determinantes de resistência ao trimetoprim descritos até à data.

Por outro lado, demonstrámos que o sucesso epidemiológico dos principais clones MRSA que circulam atualmente nos PALOP poderá ser devido à sua capacidade de sobreviver em diferentes situações de stress, incluindo uma melhor adaptação a ambientes alcalinos e a uma elevada resistência à salinidade e à dissecação. Verificámos igualmente que os principais tipos clonais MRSA do continente Africano superaram outros clones MRSA epidémicos

resistentes ao SXT, tais como o clone Brasileiro, tanto no crescimento independente como em condições de competição explicando provavelmente a ausência deste clone pandémico em África. Embora não tenhamos esgotados todas as condições de crescimento encontradas nos hospitais e em instituições de saúde, o facto dos isolados MRSA recolhidos no continente Africano serem dominantes na grande maioria dos ensaios, incluindo em condições sub-óptimas de crescimento, sugere que poderá ser uma vantagem significativa de “fitness” e explica o sucesso destes clones nos PALOP.

Palavras-chaves: *Staphylococcus aureus*, MRSA, resistência aos antibióticos, sulfametoxazol, trimetoprim, trimetoprim-sulfametoxazol, África

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ABBREVIATIONS

aa - aminoacid
agr – Arginine
ATCC – American Type Culture Collection (Manassas, USA)

bp - Base pairs

C – Cytosine
CA-MRSA – Community-associated methicillin-resistant *Staphylococcus aureus*
CC – Clonal complex
CD4 – Cluster of differentiation 4
CDC – Centers for Disease Control and Prevention (Atlanta, USA)
cfu – Colony forming units
CLSI – Clinical and Laboratory Standards Institute (Wayne, USA)

DHFR – Dihydrofolate reductase
DHPS - Dihydropteroate synthase
DNA – Deoxyribonucleic acid
DNase – Deoxyribonuclease
dNTP – Deoxynucleoside triphosphate

EDTA – Ethylenediamine tetraacetic acid

F - Phenylalanine

G – Guanine
H_2O_2 – Hydrogen peroxide

H - Histidine
HA-MRSA – hospital-associated methicillin-resistant *Staphylococcus aureus*
HCl – Hydrochloric acid

HIV – Human immunodeficiency virus

M – Molar
MH – Mueller-Hinton
MIC – Minimal inhibitory concentration
mg - Milligram
mL – Milliliter
mM – milimole
MLST – Multilocus sequence typing
MRSA – Methicillin-resistant *Staphylococcus aureus*
MSSA – Methicillin-susceptible *Staphylococcus aureus*

NaCl – Sodium chloride
nm – Nanometer

OD – Optical density

PBP – Penicillin-binding protein
PCR – Polymerase chain reaction
PFGE – Pulsed-field gel electrophoresis
pH – Potential of hydrogen
PVL – Panton-Valentine leukocidin

R - Arginine
rpm – Rotation per minute

SCC*mec* – Staphylococcal Cassette Chromosome *mec*
spa – *Staphylococcus* protein A
SMZ – Sulfamethoxazole
SXT – Trimethoprim-sulfamethoxazole
ST – Sequence type

TE – Tris-EDTA

TAE – Tris-acetate-EDTA

TMP – Trimethoprim

TSA – Tryptic soy agar

TSB – Tryptic soy broth

U - Units

USA – United States of America

V – Volt

VISA – Vancomycin-intermediate *Staphylococcus aureus*

VRSA – Vancomycin-resistant *Staphylococcus aureus*

Y - Tyrosine

µg - Microgram

µL – Microliter

µM - Micromole

CHAPTER I – INTRODUCTION

1. Staphylococcus aureus – general features

Staphylococcus was firstly identified in 1880 by the surgeon Sir Alexander Ogston, who isolated the bacterium in pus from a surgical abscess in a knee joint, and demonstrated its capacity to produce inflammation and suppuration. However, the binomial nomenclature *Staphylococcus aureus* was only introduced in 1884 by Friedrich Julius Rosenbach. The name of the genus derived from the Greek term "staphyle", which means brunch of grapes and the species epithet "*aureus"* that comes from the Latin word for gold, due to its golden pigmentation (72, 101).

S. aureus is a Gram-positive coccus nonmotile, non-spore forming, facultative anaerobic, and member of the Micrococcaceae family. When grown on agar plates, *S. aureus* appears as large round, golden yellow colonies and as grape-like cluster when viewed under the microscope (84). It is catalase, coagulase and DNase positive, ferments mannitol and presents a low G + C content (30-38%). This microorganism is extremely versatile, tolerating high salt concentration (up to 1.7 M), extreme temperatures (up to 50ºC), different pH (4.8 to 9.4) and drying conditions (72).

S. aureus is an ubiquitous bacterium, part of the human microbiota, with up to one third of normal healthy population being carriers (124). This microorganism can be found in different parts of the body, however anterior nares are the primary ecological niche in humans (124). *S. aureus* is also known as an opportunistic pathogen when the immune system becomes compromised, and it is a major cause of infections worldwide. It is responsible for a wide array of diseases ranging from pyogenic skin infections to complicated life-threatening diseases and toxinoses (115, 118).

2. Evolution of resistance in *Staphylococcus aureus*

In the pre-antibiotic era, the mortality of patients with *S. aureus* bacteremia exceeded 80% and over 70% developed metastatic infections (109). The introduction of penicillin in 1941 into clinical practice dramatically improved the prognosis of patients infected with pathogenic *S. aureus.* However, one year later, in 1942, the first penicillin-resistant staphylococci were recognized in the hospital and subsequently in the community (9, 94). At the end of 1960, more than 80% of staphylococcal isolates were resistant to penicillin due to the acquisition of a plasmid borne-β-lactamase capable of hydrolyzing and destroying the penicillin molecule before it reaches the intracelular target. Nowadays, the great majority of *S. aureus* strains are β-lactamase producers and penicillin has almost become useless (15, 24).

Methicillin, initially called celbenin, was the first semisynthetic penicillinase-stable β-lactam antimicrobial, which was introduced into clinical practice in 1960. However, in 1961, one year after its introduction, the first methicillin-resistant *S. aureus* (MRSA) isolates were reported in England (66). Its resistance was due to the introduction of a large piece of foreign DNA, called Staphyloccocal cassette chromosome *mec* (SCC*mec*), which carries the central element of methicillin resistance – the *mecA* gene (64). The *mecA* gene encodes an extra penicillin-binding protein (PBP), the PBP2a, characterized by low affinity to virtually all β-lactam antibiotics, including cephalosporins and carbapenems.

During the following decades, *S. aureus* has sequentially acquired and developed resistance to a wide variety of antimicrobial agents, either by mutations in genetic determinants encoding target proteins, or through horizontal acquisition of antibiotic resistance genes. Nowadays, the increasing rates of multidrug resistance pose a serious challenge for the treatment of MRSA infections, limiting the therapeutic to the "last-resort" antimicrobials such as vancomycin, linezolid, quinupristin-dalfopristin and daptomycin (103). Once again, the emergence of resistance to linezolid, quinupristin-dalfopristin and daptomycin was reported shortly after their clinical introduction (78, 120, 123). Furthermore, in 1997, the first *S. aureus* clinical isolate with low-level resistance to vancomycin (VISA) was reported in Japan (59), followed by the emergence of vancomycin-resistant *S. aureus* (VRSA) isolates in 2002 (22). Several recent reports of infections caused by VRSA (8, 50, 75) led to concerns that, in the near future, staphylococcal infections may no longer be treatable (25). Although antimicrobial

resistance is not imperative for survival of *S. aureus*, it undoubtedly contributes to the success of the bacterium under the selective pressure of antimicrobial chemotherapy (25).

3. Molecular evolution of MRSA: from the hospital to the community

After the first report of MRSA in England, these resistant isolates began to spread, reaching epidemic proportions in several European countries in the 1960s and in other parts of the world, such as Australia, Japan and the USA in the late 1970s (41).

From 1961 until 1993, MRSA isolates were restricted to healthcare facilities, being considered hospital-associated MRSA (HA-MRSA). However, since the late 1990s, there was a significantly increase in the prevalence of MRSA isolates in children and adults with no contact with hospital settings, leading to the emergence of community-associated MRSA (CA-MRSA) (41). The first CA-MRSA emerged among aboriginal communities in Australia in 1993 (121) and nowadays CA-MRSA has been described worldwide (80, 99). CA-MRSA strains tend to be more virulent due to the presence of various virulence factors, namely the Panton-Valentine leukocidin, show a faster growth, are frequently susceptible to non-β-lactams and have a lower degree of β-lactam resistance (89). Nowadays, the difference between HA-MRSA and CA-MRSA is beginning to fade; major HA-MRSA clones have been described in the community and CA-MRSA clones are being the cause of nosocomial outbreaks (37).

MRSA has been considered as a globally important pathogen and it remains a major cause of healthcare associated infections not only in the developed world, but in developing regions as well where the human immunodeficiency virus (HIV), malaria, malnutrition, crowded living conditions, high temperatures and humidity additionally contribute to the increased risk of bacterial infections (12, 122).

4. MRSA clonal distribution

The worldwide spread of MRSA is driven by the dissemination of a number of clones with a specific genetic background (41). MRSA clones are usually defined by the combination of their multilocus sequence type (MLST) and the SCC*mec* type they carry (*e.g*. ST239-SCC*mec* III, abbreviated as ST239-III) (44).

Some of the major international HA-MRSA clones include the Brazilian/Hungarian (ST239-III), New York/Japan (ST5-II), Pediatric (ST5-VI and ST5-IV_a), EMRSA-15 (ST22-IV_h) and EMRSA-16 (ST36-II).

ST239-III is one of the most successful and persistent HA-MRSA clones; it is multidrug-resistant and accounts for 90% of HA-MRSA in the Asiatic continent (58). This clone currently represents one of the major lineages in South America (5, 98), the major clone in Asia (6, 28), represents 43% of the MRSA isolates in Australia (34), and is also present in Europe (55) and Africa (1). Likewise, the New York/Japan (ST5-II) and the Pediatric (ST5-VI and ST5-IV_a) clones, belonging to Clonal Complex (CC) 5, are globally disseminated in North (97) and South America (98), Europe (55) and Asia (74), causing serious infections in healthcare settings and in the community. The EMRSA-15 (ST22-IV_h) and EMRSA-16 (ST36-II) clones, firstly identified in England (82, 87), are nowadays predominant clones in many European countries in the hospital (2, 46, 55) and community settings (114), but only sporadically found in the United States (79, 125).

Regarding CA-MRSA clonal lineages, the major lineages are the USA300 (ST8-IV_a), European (ST80-IV_{NT} and ST80-IV_c), Southwest/Pacific (ST30-IV), Queensland (ST93-IV) and ST59-IV clones (40). ST80-IV is widely spread in Europe, Northern Africa, Singapore and the Middle-East (37). USA300 clone (ST8-IV_a) is predominately disseminated in the United States (127), while the Southwest/Pacific clone (ST30-IV) is mainly found in Oceania together with ST93-IV and in Asia together with ST59-IV (60).

On the other side, the scenario in the African continent appears to be quite different. It was shown that the predominant hospital-associated epidemic clones, such as Brazilian/Hungarian, EMRSA-15 and EMRSA 16 clones, were reported only sporadically in the African continent (81). However, MRSA assigned to CC5, such as the Pediatric clone (ST5-

IV_a), are widely spread across Africa, namely in Portuguese speaking African countries (1, 33). In addition, CA-MRSA clones, such as the West Australia MRSA-2 clone (ST88-IV_a) and ST8-IV_a clone, are highly prevalent in the African continent, in both hospital and community settings (1, 33).

5. Antifolate antibiotics: sulfonamides and trimethoprim

Antifolate antimicrobials are the compounds that act on the bacterial folic acid biosynthesis pathway, inhibiting its final production. Folic acid derivatives, such as tetrahydrofolate, is essential for bacterial DNA synthesis and thus for its replication. The two most known antifolate compounds with antibacterial properties are sulfonamides and trimethoprim (13).

Sulfonamides were the first class of antimicrobial agents with a selective effect on bacteria that could be used systemically against bacterial infections. In 1932 Gerhard Domagk showed that mice infected intraperitoneally with *Streptococcus pyogenes* could be protected from peritonitis by the chemically synthetized Prontosil (4-sulfonamide-2', 4'-diaminoazobenzene) (108). Sulfonamides were introduced into clinical practice in 1935 and, since then, they have been used extensively for different clinical indications. The medium long-acting sulfonamide, sulfamethoxazole (SMZ) – Figure 1, remains the most useful member of this class of antimicrobial agents. Nevertheless, sulfonamides can cause serious side effects, including significant hypersensitivity or toxic reactions, and are not used very frequently as a single drug. In addition, sulfonamides are the most important drugs to be considered causes of blood dyscrasias. With the introduction of new and safer antibacterial agents, these side effects have reduced the attractiveness of sulfonamides in most developed countries (62).

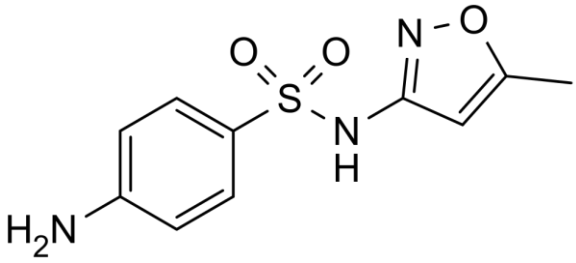

Figure 1 – Chemical structure of Sulfamethoxazole (SMZ).

Trimethoprim (TMP), a synthetic antimicrobial agent (Figure 2), was initially used for the treatment of infections in 1962 (63). Ten years later, it was firstly introduced as prophylaxis for urinary tract infections, in Finland, and then for the treatment of patients with acute urinary tract infections in 1979. TMP presents fewer side effects than sulfonamides, however rashes and other hypersensitivity reactions have been reported, especially among patients with HIV (62).

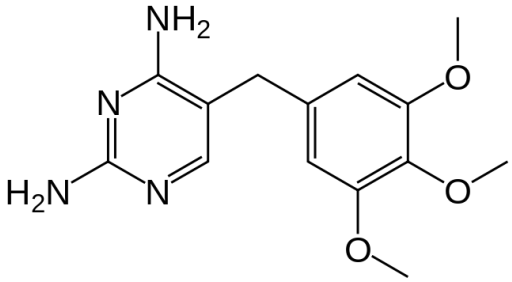

Figure 2 – Chemical structure of Trimethoprim (TMP).

5.1. Use of trimethoprim and sulfamethoxazole

Use of TMP in combination with sulfonamides was registered for clinical use in 1968 (19, 63). Currently, the combination of trimethoprim-sulfamethoxazole (SXT), also called co-trimoxazole, is used as a 5:1 fixed-dose that produces a 20:1 plasma concentration due to the greater volume distribution of TMP compared with that of SMZ. SXT is effective for empirical treatment of skin and soft tissue infections caused by CA-MRSA in Africa, but also in Europe and North America (38, 112).

The drastic stagnation in the development of novel antibacterial chemotherapies increasingly forces infectious diseases practitioners to rediscover “old antibiotics”, such as antifolate antagonists (17, 21). The recent observation that SXT, in combination with rifampicin, is a non-inferior treatment to linezolid for the treatment of severe staphylococcal infections, illustrates the renewed interest in, and potential of antifolate compounds (57).

5.2. Mechanism of action of SMZ and TMP

The combination of the two antifolate compounds, SXT, acts synergistically by inhibiting two essential enzymes in the bacterial folic acid biosynthesis pathway: SMZ inhibits dihydropteroate synthetase (DHPS), which catalyzes the formation of dihydropteroic acid from *para*-aminobenzoic acid and pteridine; in the subsequent step of the pathway, TMP inhibits dihydrofolate reductase (DHFR), essential for the formation of tetrahydrofolate from dihydrofolate – Figure 3 (62).

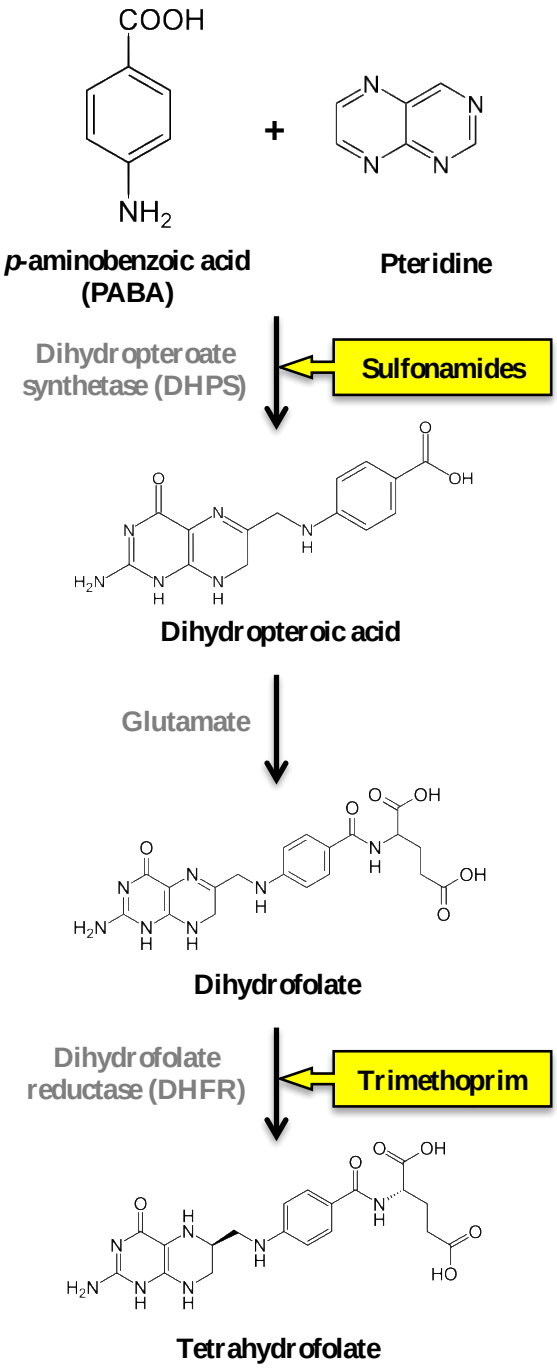

Figure 3 – Staphylococcal folic acid biosynthesis pathway and locals of inhibition of sulfonamides and trimethoprim antibiotics.

5.3. Resistance to sulfonamides

Sulfonamides resistance is usually conferred by non-synonymous mutations in the gene encoding the dihydropteroate synthase (DHPS), which alters the amino acid sequence of this protein (62). In 1997, the DHPS gene from *S. aureus*, designated *folP* or *dpsA*, was cloned for the first time, sequenced and the protein expressed in *Escherichia coli*. Southern-blot analysis of *S. aureus* chromosomal DNA indicated that there is only one gene encoding DHPS, the *S. aureus* DHPS (SaDHPS) (56).

Several clinical MRSA isolates were studied showing that resistance to sulfonamides in *S. aureus* is only due to mutations in DHPS gene, and not to transferable markers, since the strains remained resistant when cured of their plasmids (116). In this context, the analysis of mutations in the chromosomal *folP* gene could discern four different groups, based upon the amino acid changes, and implicate as many as 14 residues scattered over the surface of the protein (56, 108).

5.4. Resistance to trimethoprim

Resistance of *S. aureus* to TMP was first reported in 1980s (77). Two genetic mechanisms are known: (i) resistance genes that encode variant DHFRs that are less sensitive to TMP than intrinsic DFHR [*S. aureus* DHFR (SaDHFR)], located on exchangeable genetic elements; and (ii) mutations on the chromosomal DHFR gene (*dfrB*) (36, 68, 100, 104). The acquired *dfr* gene variants mediate high-level resistance to TMP (MIC > 512 μg/mL). To date, three of such genes have been identified in *S. aureus*: *dfrA*, *dfrG* and *dfrK*, all of them being carried in plasmids (68, 100, 104). In contrast, the mutation of the instrinsic *dfrB* gene in *S. aureus*, a single functional mutation at position 98 (F99Y), confers intermediate-level TMP resistance (MIC ≤ 256 μg/mL) (36).

Until recently, the single *dfrB* F99Y mutation and the acquired *dfrA* gene are considered to be the key determinants of TMP resistance in *S. aureus* isolated from humans (48, 49, 65,

88). In contrast, *dfrG* gene was perceived as being rare among *S. aureus* isolates from human origin (88), but plays an important role in particular clones of *Staphylococcus pseudointermedius* (93) and *S. aureus* of animal origin (10). *dfrG* gene was reported for the first time among MRSA isolates in Thailand in 2005 (104), and five years later, an outbreak of MRSA carrying this gene occurred in a hospital in London (61). More recently, a widespread prevalence of *dfrG* in *S. aureus* isolates causing human infections in the sub-Saharan Africa and its abundance in imported *S. aureus* from ill returning travelers from Africa to Europe, suggest an African origin of this gene (85). The third DHFR variant, *dfrK* gene, has been identified predominantly in livestock-associated MRSA belonging to Sequence Type (ST) 398 from Europe and only sporadically in humans (10, 35, 47, 67).

Comprehensive studies on the relative contribution of the different TMP resistance genes in human *S. aureus* isolates are scarce, namely from low resource countries where it is widely used, and represent major implications for the development of new antifolate antibiotics.

5.5. Resistance to SXT and its global distribution

S. aureus isolates fully resistant to SXT were firstly identified in the 1980s (63). SXT resistance rates among *S. aureus* vary considerably depending on the location, as well as the time period. In North America, very low resistance rates have been reported in clinical *S. aureus* isolates, ranging from 0 to 7% (69, 96, 113). In addition, a recent report showed a decreased of SXT-resistant MRSA strains in the United States over the years (16). In Europe, low-to-medium SXT resistance levels have been generally observed in most of the countries (111); 1% in Spain (76), 1.1% in Turkey (42) and Greece (110), and 14.5% in Italy (31). Portugal is one of the exceptions, where the reported rate of SXT resistance reached 67% of the staphylococcal isolates (7). On the other hand, high levels of SXT resistance among MRSA isolates have been observed in South America (up to 100%) (5, 106), as well as in the Asiatic continent, namely in Taiwan (89%) and China (21%) (6).

Clonal outbreaks of MRSA resistant to SXT have been reported; of these, the globally disseminated hospital-associated Brazilian/Hungarian clone and variants (ST239/241) has been described to be usually associated with SXT resistance (53). The high prevalence of ST239 in Asia, South America and Portugal (58) explains the high resistance rate of SXT in these geographical locations.

Although the prevalence of ST239 is less frequent in the African continent (1) and completely absent in Portuguese-speaking African countries (33), high levels of SXT resistance were also reported in this continent associated to ST5-IV_a, ST88-IV_a and ST8-IV_a clones. In fact, up to 55% of colonizing and 72% of clinical *S. aureus* isolates from Africa, are resistant to SXT (18, 33, 85, 90, 91). The frequent use of this inexpensive antifolate compound as first-line option for the treatment and prevention of infections in the African continent, might explain the high resistance rates (90). Moreover, prophylactic use of SXT in resource-constrained areas is widely recommended for HIV-infected or HIV-exposed children, to everyone with CD4 cell counts bellow 350, and to those with stage III and IV disease (126), which also partly explains the increased SXT resistance in Africa. Therefore, monitoring the genetic basis of SXT resistance in this continent is essential to anticipate its further spread.

6. Hetero-resistance in *S. aureus*

In board terms, hetero-resistance is defined as resistance to certain antibiotics expressed by a subset of a microbial population that is generally considered to be susceptible to these antibiotics, according to traditional *in vitro* susceptibility testing. It could be a tool for natural evolution to drug resistance since it provides an opportunity to the bacteria to explore the possibility of growth in the presence of antibiotics before acquisition of resistance by the major proportion of the microbial population (45).

This phenomenon has been described in a wide variety of microorganisms, but much attention has been directed towards its expression in *S. aureus.* Firstly described in 1994 in MRSA isolates with heterogeneous resistance to methicillin (102), reports of this phenomenon have been restricted to β-lactams and glycopeptides until now (39, 45, 71, 102).

7. Aim of the study

The aim of the present study is to provide new insights on the high rates of SXT-resistant *S. aureus* isolates in the African continent (i) by determining for the first time the prevalence of the different TMP resistance markers in Portuguese-speaking African countries, namely São Tomé and Príncipe, Angola and Cape Verde and (ii) by evaluating the fitness and resistance to chemical stresses of the three major MRSA clones circulating in these African countries, all SXT-resistant. Furthermore, we will evaluate the success of these clones by comparing to the Brazilian MRSA (ST239 or variant ST241), also resistant to SXT, which is widely spread all over the word and prevalent in some other countries in Africa but not detected so far in these three Portuguese-speaking African countries.

CHAPTER II – MATERIALS AND METHODS

1. Bacterial collection

For this study, we selected a total of 122 *S. aureus* isolates previously found resistant to SXT (85 MRSA and 37 MSSA), from three Portuguese-speaking African countries (Angola, São Tomé and Príncipe, and Cape Verde), recovered from nasal swabs between 2010 and 2014 (33) – Tables 1 and 2. The isolates were chosen from an initial collection of 507 isolates, in order to represent the proportion of each clonal type in the three countries included in this study.

Table 1 – Genotypic properties of SXT-resistant MRSA isolates from Angola, São Tomé and Príncipe, and Cape Verde (33).

Clonal type					No. of isolates			
PFGE[1]	***spa* types[2]**	**ST[3]**	**SCC*mec*[4]**	***agr*[5]**	**Angola**	**São Tomé and Príncipe**	**Cape Verde**	**TOTAL**
A	t105/t311/ t11657	5	IVa	II	34	2		36 (42.4%)
B	t186/t325/ t786/ t1814/ t1951/t3869	88	IVa	III	12	6	2	20 (23.5%)
C	t064/t104/ t451/t1771	8	IVc/IVd/IVg/V	I	7	9		16 (18.8%)
D	t3092	72	V	I	4			4
J	t091	789	V	I	1			1
U	t6065	5/2629	V	II	2			2
X	t6278	30	V	III	1			1
ABB	t002	5	VI	II			2	2
AQ	t1476	8	VII	I	3			3
TOTAL					**64**	**17**	**4**	**85**

[1] PFGE – Pulsed-field gel electrophoresis
[2] *spa – Staphylococcus* protein A
[3] ST – Sequence type
[4] SCC*mec* – Staphylococcal cassette chromosome *mec*
[5] *arg* - Arginine

Table 2 – Genotypic properties of SXT-resistant MSSA isolates from Angola, São Tomé and Príncipe, and Cape Verde (33).

Clonal type				No. of isolates			
PFGE[1]	***spa* types[2]**	**ST[3]**	***Agr*[4]**	**Angola**	**São Tomé and Príncipe**	**Cape Verde**	**TOTAL**
C	t064/t1774/t13119	8	I	1	3		4
K	t084/t774	15	II	5	9	7	21
M	t355	152	I	1	1		2
N	t008/t1476	8	I	2			2
O	t159/t314/t2304	121	IV	1	4		5
W	t349	25	I	1			1
X	t9118	30	III	2			2
TOTAL				**13**	**17**	**7**	**37**

[1] PFGE – Pulsed-field gel electrophoresis
[2] *spa* – *Staphylococcus* protein A
[3] ST – Sequence type
[4] *arg* – Arginine

In addition, 18 SXT-resistant MRSA isolates representative of major clones widely spread in South America (Argentina, Brazil, Chile and Uruguay), Portugal and Taiwan, were also included (Table 3), reaching a total of 140 *S. aureus* isolates (4-6).

Table 3 – Genotypic properties and origin of SXT-resistant MRSA isolates from other continents belonging to the Brazilian clone or variant (4-6).

ST[1]	**Origin**	**Period of isolation**	**SCC*mec*[2]**	**No. of isolates**	**TOTAL**
ST239	Brazil	1996-1998	III	3	**18**
	Argentina	1995-1996	III	2	
	Uruguay	1996-1998	III	3	
	Chile	1997-1998	III	3	
	Portugal	1991-1997	III	4	
	Taiwan	1998-1999	III	1	
ST241	Taiwan	1998-1999	III	2	

[1] ST – Sequence type
[2] SCC*mec* – Staphylococcal cassette chromosome *mec*

2. Antimicrobial susceptibility testing and minimum inhibitory concentration (MIC)

Antimicrobial susceptibility testing was performed for all isolates by the disk diffusion method for SXT, TMP and SMZ, according to standard published Clinical Laboratory Standards Institute (CLSI) guidelines (30). An isolate was considered (i) susceptible when the zone diameter (≥16 mm for SXT and TMP; ≥17 mm for SMZ) was in accordance to the susceptible criteria and there was a clear inhibition zone; (ii) hetero-resistant when there were distinct colonies growing or growth within a zone of inhibition creating a double zone; and (iii) fully resistant when the zone diameter (≤10 mm for SXT and TMP; ≤12 mm for SMZ) was in accordance to the resistance criteria and there were no colonies or growth within the inhibition zone.

In addition, the minimum inhibitory concentrations (MIC) for TMP and SMZ were determined for all isolates by the broth microdilution method following Clinical Laboratory Standards Institute (CLSI) guidelines (30) and in parallel by incubating and reading with a TECAN Infinite 200 Pro reader (Tecan group Ltd., Männedorf, Switzerland). Since the results were comparable with the two methodologies, the MICs for TMP and SMZ were subsequently performed for all isolates with the TECAN reader. Briefly, overnight bacterial cultures were adjusted to an optical density of 0.085 OD_{620}, and 5 µL of each adjusted cell suspension was added to 150 µL of fresh MH, containing the appropriate antibiotic concentration. The plates were incubated in a TECAN Infinite 200 Pro reader (Tecan group Ltd., Männedorf, Switzerland) for 15 h at 37ºC with shaking (180 rpm). Measurements were performed every 20 min at 595 nm. Positive (without antibiotic/with inoculum) and negative (without antibiotic/without inoculum) controls were included for each isolate. We considered that there was bacterial growth when the optical density after 15h reached at least 0.2. The MIC for SXT was determined by Etest (AB BioMérieux, Solna, Sweden) for all isolates previously identified as SXT-resistant by the disk diffusion method.

3. Total DNA extraction

Four to five colonies of cultures grown overnight on Tryptic Soy Agar (TSA) were incubated into 20 µL TE 1X (10 mM Tris, 1 mM EDTA, pH 8) with 10 mg/mL of lysostaphin

for 30 min performing cell lysis, followed by an enzyme denaturation at 95ºC for 15 min. The mixture was centrifuged at 13 000 rpm for 5 min and the supernatant was recovered.

4. Detection of TMP resistance genes

Isolates were screened for known *S. aureus dfr* genes by conventional PCR (Table 4) in a T1 Thermocycler (Biometra, Germany). The presence of *dfrA* and *dfrG* genes was determined for all isolates. The isolates without amplification of *dfrA* and *dfrG* genes were subsequently screened for the presence of *dfrK*. Isolates for which there was no amplification of the acquired *dfr* genes were subsequently analysed for their intrinsic *dfrB* gene. The following isolates were used as positive controls: ANG880 for *dfrA*, ANG17A for *dfrG*, FVL88.1 for *dfrK* and ATCC25923 for *dfrB* gene.

The DHFR-encoding *dfrB* was also sequenced and then aligned with strain ATCC25923, using DNASTAR Lasergene 8 Softwares: EditSeq, SeqMan, Protean and MegAlign (DNASTAR, Madison, WI, USA) to identify possible non-synonymous mutations.

For each gene, the PCR mixture contained 1X PCR Buffer with 1.5 mM $MgCl_2$, 40 μM of each deoxynucleotide triphosphate (dNTP), 0.4 μM of each *primer*, 1.25 U of Taq DNA polymerase (GoTaq – Promega, Madison, USA) and 2 μL of chromossomal DNA template in a final volume of 50 μL. PCR produts (10 μL) were resolved in 1.5% Seakem LE agarose (Cambrex Bio Science Rockland, USA) for *dfrG*, *dfrK* and *dfrB* genes and 2% for *dfrA* gene in 1X Tris-acetate-EDTA (TAE) buffer at 80V for 60 min and visualized with ethidium bromide.

Table 4 – Primers nucleotide sequences, product size and PCR conditions of the genes amplified in this study.

Gene	Primer nucleotide sequences (5' - 3')	Product size	PCR conditions	References
dfrG	**F:** TGCTGCGATGGATAAGAA **R:** TGGGCAAATACCTCATTCC	405 bp	94ºC – 4 min 94ºC – 1 min 57ºC – 30 s 72ºC – 1 min 30 x 72ºC – 4 min	10, 85
dfrA	**F:** CACTTGTAATGGCACGGAAA **R:** CGAATGTGTATGGTGGAAAG	270 bp		10, 85
dfrK	**F:** GCTGCGATGGATAATGAACAG **R:** GGACGATTTCACAACCATTAAAGC	321 bp	94ºC – 4 min 94ºC – 30 s 49ºC – 30 s 72ºC – 1 min 35 x 72ºC – 5 min	47
dfrB	**F:** AATTGTGTTAAATTAAAGATAACTT **R:** TAAGTATTCTTTAGATAAATCGGAT	572 bp	94ºC – 4 min 94ºC – 1 min 43ºC – 30 s 72ºC – 1 min 35 x 72ºC – 4 min	85

5. Fitness experiments

Fitness experiments were performed as previously described (11, 73) with minor alterations detailed below.

A total of five SXT-resistant MRSA isolates were selected, which included: (i) representative strains of the three major MRSA clonal types in São Tomé and Príncipe - STP33 (ST88-SCC*mec* IV_a), STP46A (ST5-SCC*mec* IV), STP151 (ST8-SCC*mec* IV_g); and (ii) representatives of the pandemic Brazilian clone or related: HSJ216 (ST239-SCC*mec* III), and TAW10 (ST241-SCC*mec* III).

5.1. Growth curves

For independent growth measurement, bacteria grown overnight in Tryptic Soy Broth (TSB) were diluted into 200 µL of fresh TSB to an initial concentration of 0.05 OD_{620} in BrandPure Grade S plates. The cultures were incubated in a TECAN Infinite 200 Pro reader (Tecan group Ltd., Switzerland) for 20 h at 37ºC with shaking (180 rpm). Readings were taken every 20 min at 595 nm. All isolates were tested with at least three independent replicates.

5.2. Competition assays

Isolates STP33, STP46A and STP151 were tested against HSJ216, and STP151 was also tested against TAW10. A 1:1 inoculum of each pair of strains was co-cultured for 24 h. For that, overnight bacterial cultures were diluted in TSB to 0.085 OD_{620}. 50 µL of the adjusted cell suspension of each of the two tested strains were added to 5 mL of fresh TSB medium and incubated at 37ºC with shaking. Appropriate dilutions of samples were plated in duplicate onto selective plates (TSA with 5 µg/mL gentamicin), and non-selective TSA plates at 0 h, 2 h, 4 h, 6/7 h and 24 h after inoculation. Viable colony counts on the selective plates indicated the bacterial levels (cfu/mL) of HSJ216 or TAW10, since these isolates were resistant to

gentamicin, while the difference between colony counts on selective and non-selective plates indicated the bacterial levels (cfu/mL) of the tested strain (STP33, STP46A or STP151).

The initial ratio [$N_S(0)/N_R(0)$] and final ratio [$N_S(24)/N_R(24)$] were calculated and the relative fitness (F) was obtained with the following formula: $F=\ln[N_S(24)/N_S(0)]/\ln[N_R(24)/N_R(0)]$, where $N_S(t)$ represented the bacterial levels (cfu/mL) of STP33, STP46A or STP151 and $N_R(t)$ the bacterial levels (cfu/mL) of HSJ216 or TAW10, at 0 and 24 h.

5.3. Survival assays

For survival experiments, 100 µL of overnight bacterial cultures were suspended in 900 µL of TSB. 100 µL of each suspension was plated onto five different empty sterile Petri dishes, manually shaken individually for approximately 1 min, and left closed on a shelf to dry. Samples were taken at 6 h, 24 h, 3 days, 5 days and 7 days, by adding 900 µL of saline solution and shaking manually for approximately 20 s. The saline solution was left on the closed Petri dish for 5 min, and then appropriate dilutions were plated onto TSB, incubated for 24 h, and viable colonies were counted.

The percentages of bacterial surviving until the final time points of 6 h or 7 days were calculated, as well as the average daily death rate (K) at day 1 (24 h), using the following formula: $K= 2.3 \times [(B_0-B_X)/1]$, where B_x is the $\log_{10}$ cfu at day X from the time of inoculation.

6. Resistance to chemical stresses

The following experiments were performed as previously described (103) with minor alterations detailed below.

6.1. Growth under stress conditions

For experiments of stress conditions, overnight cultures grown in TSB were diluted into 200 µL of fresh TSB to an initial concentration of 0.02 OD_{620} in 96-well BrandPure Grade S

plates. Simultaneously, bacteria were grown under stress conditions through appropriate modifications of TSB: 8.8 mM H_2O_2, 4% (v/v) ethanol, low pH (pH 4.5), high pH (pH 10) and 2.5 M NaCl. Growth was followed spectrophotometrically in a TECAN Infinite 200 Pro reader (Tecan group Ltd., Switzerland) at 37ºC with shaking (180 rpm). Readings were taken at 595 nm every 20 min for 18 h. All isolates were tested with three independent replicates.

6.2. Whole-cell autolysis assays

For bacterial autolysis assays, strains were grown overnight in TSB at 37ºC with shaking (180 rpm). After washing cells twice with cold water, cells were resuspended to an initial concentration of 1.2 OD_{620}, in a 40 mL final volume of 0.05 M Tris/HCl buffer, pH 7.2, containing 0.05% Triton X-100. The suspensions were incubated at 37ºC with shaking (180 rpm), and readings were taken every 30 min for 6 h.

CHAPTER III – RESULTS

1. Antibiotic resistance and screening of TMP resistance genes

The 140 *S. aureus*, initially selected for being previously reported as resistant to SXT, were analysed.

1.1. Antimicrobial susceptibility testing and MIC

All 122 isolates recovered from the African continent were confirmed to be full resistant to TMP, presenting a MIC ≥ 1024 µg/mL, with the exception of one isolate from São Tomé and Príncipe, that presented a MIC of 256 µg/mL – Annex 1/Table 1.

However, full resistance to the combination of TMP and SMZ (resistance to SXT) was only found in 20 isolates (16.4%). The MIC for SXT ranged from 6 to >32 µg/mL, and from 1024 to 2048 µg/mL for SMZ (Figure 4). Interestingly, these isolates (16 MRSA and 4 MSSA) belonged to the same clonal type (PFGE C-ST8-SCC*mec* $IV_c/IV_d/IV_g/V$) and were recovered from two different countries (Angola and São Tomé and Príncipe) – Annex 1/Table 1.

A **B**

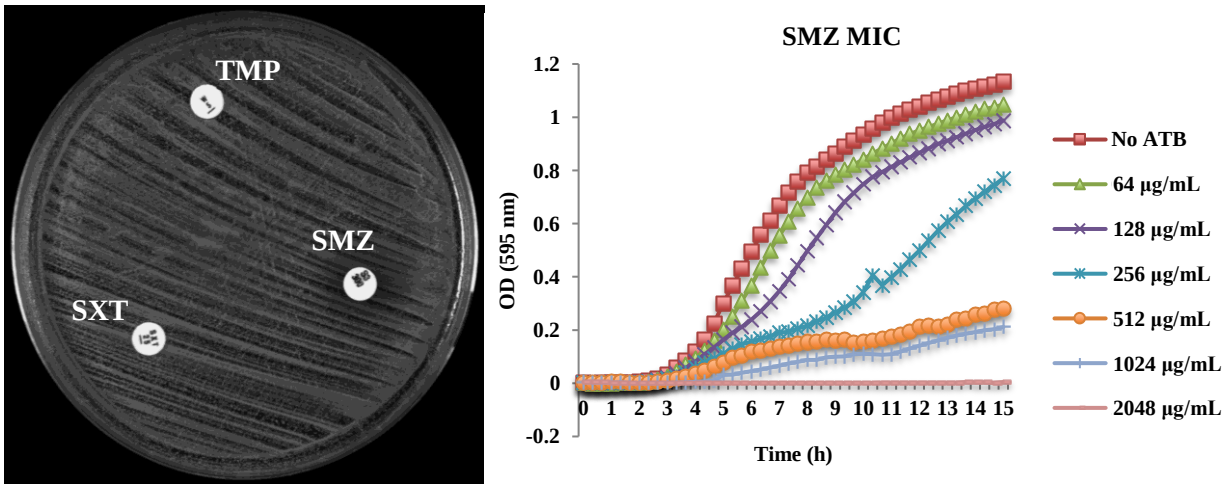

Figure 4 – Antimicrobial susceptibility testing of an isolate fully resistant to TMP, sulfonamides and SXT by A) disk diffusion test; B) MIC determination for SMZ determined with the Tecan spectrophotometer.

Interestingly, the remaining 102 African isolates (83.6%) presented a double halo for SXT showing some growth inside (Figure 5A). Of these, 58 isolates were considered hetero-resistant for sulfonamides presenting also double halo phenotype (Figure 5A) and showing a MIC for SMZ ranging between 1024 to 2048 µg/mL (Annex 1/Table 1). The SMZ MIC determination with the Tecan equipment showed that only a small proportion of the original culture could grow even in the lower concentration of antibiotic (Figure 5B). Accordingly, the hetero-resistance to sulfonamides in the presence of full TMP resistance was not sufficient to render *S. aureus* isolates fully resistant to SXT - Figure 5 and Table 1/Annex 1.

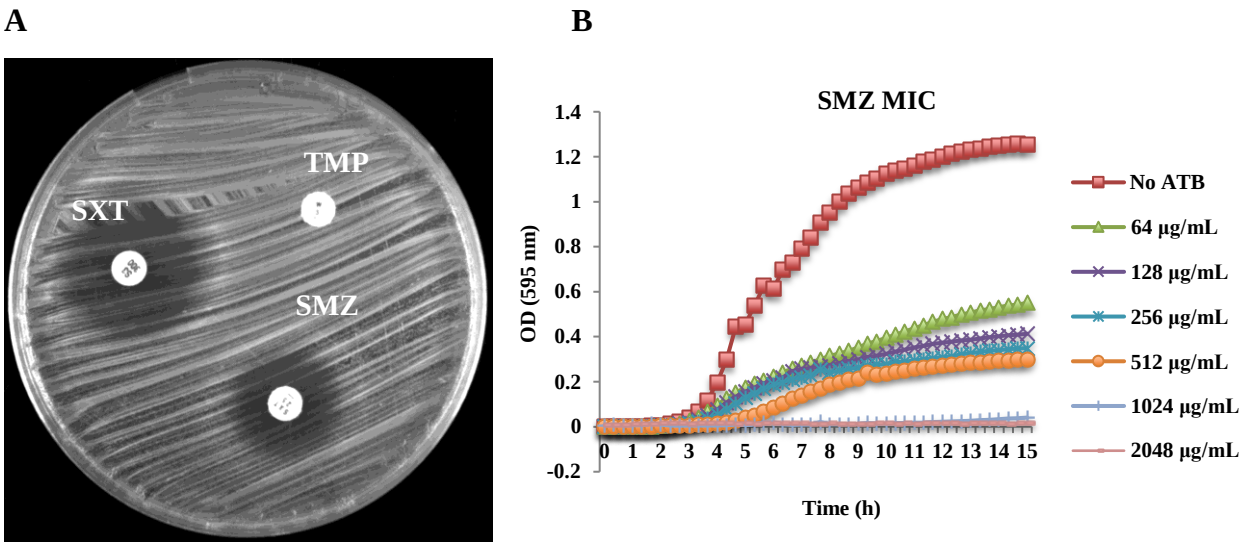

Figure 5 – Antimicrobial susceptibility testing of an isolate fully resistant to TMP and hetero-resistant to sulfonamides and SXT by A) disk diffusion test; B) MIC determination for SMZ determined with the Tecan spectrophotometer.

The remaining 44 isolates hetero-resistant for SXT were fully susceptible to sulfonamides, presenting a clear halo on the disk diffusion test and the MIC for SMZ ranged from ≤64 to 256 µg/mL – Figure 6 and Annex 1/Table 1.

A **B**

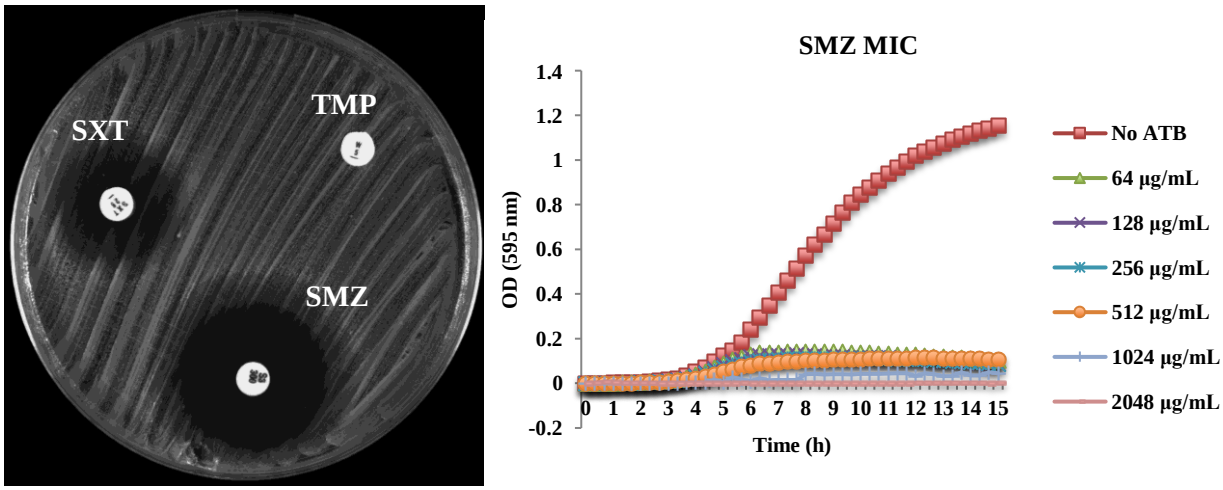

Figure 6 – Antimicrobial susceptibility testing of an isolate fully resistant to TMP, hetero-resistant to SXT and susceptible to sulfonamides by A) disk diffusion test; B) MIC determination for SMZ determined with the Tecan spectrophotometer.

All 18 MRSA isolates recovered from South America, Portugal and Taiwan were full resistant to TMP, SMZ and SXT presenting MICs ranging from 256 to >1024 µg/mL for TMP, from 1024 to 2048 µg/mL for SMZ and from 24 to >32 µg/mL for SXT – Table 1/Annex 1.

1.2. Detection of TMP resistance genes

The 122 African isolates were tested for the presence of TMP resistance genes *dfrG* and *dfrA*. The *dfrG* gene was the most prevalent gene (81.1%) and was detected in 99 isolates (62 MRSA and 37 MSSA). It was not clustered to any particular *S. aureus* clone, being present in almost all clonal types. The *dfrA* gene was detected in 27 MRSA isolates (22.1%). It was mainly associated with clones PFGE B-ST88-SCC*mec* IVa (n=20, 100%) and PFGE ABB-ST5-SCC*mec* VI (n=2, 100%) but was also found among three other clonal types: PFGE AQ-ST8-SCC*mec* VII (n=2, 66.7%) and PFGE C-ST8-SCC*mec* IVd (n=3, 15%). Although the overwhelming majority of the isolates presenting *dfrG* gene lacked *dfrA* gene, four MRSA isolates belonging to clonal types B (n=2) and AQ (n=2) contained both genes. Since all

African isolates harboured *dfrG* or *dfrA* genes, the screening for the presence of *dfrK* and *dfrB* was not performed in these isolates – Table 1/Annex 1.

Regarding the 18 MRSA isolates from other continents, only the two ST241 isolates from Taiwan presented *dfrG* gene; *dfrA* gene was only found in one isolate from Portugal. The presence of *dfrK* gene was screened in the remaining 15 isolates but none was found positive. However, 10/15 isolates harboured *dfrB*, which was further sequenced, presenting three synonymous and two non-synonymous mutations (F99Y and R150H) in all isolates. For each non-synonymous mutation, the aminoacid was replaced by another aminoacid of the same family: phenylalanine (F) and tyrosine (Y) belonged to the aromatic R group, and Arginine (R) and Histidine (H) belonged to the positively charged R group. A total of five isolates, recovered from Brazil (n=2), Argentina (n=1), Taiwan (n=1) and Portugal (n=1) did not amplify any of the TMP resistance determinants described so far.

2. Comparison of major SXT-resistant MRSA clonal types recovered from São Tomé and Príncipe against major SXT-resistant MRSA ST239/241 international clones

2.1 Fitness experiments

Independent growth experiments showed that the three African isolates (STP33, STP46A and STP151) had shorter lag phases and higher growth rates than TAW10 and HSJ216 - Figure 7.

There was no significant difference between the three isolates recovered from São Tomé and Príncipe. Therefore, the full resistance to SXT and sulfonamides of STP151 did not seem to cause any advantage or any fitness cost to the strain, when compared to the hetero-resistant isolates STP33 and STP46A.

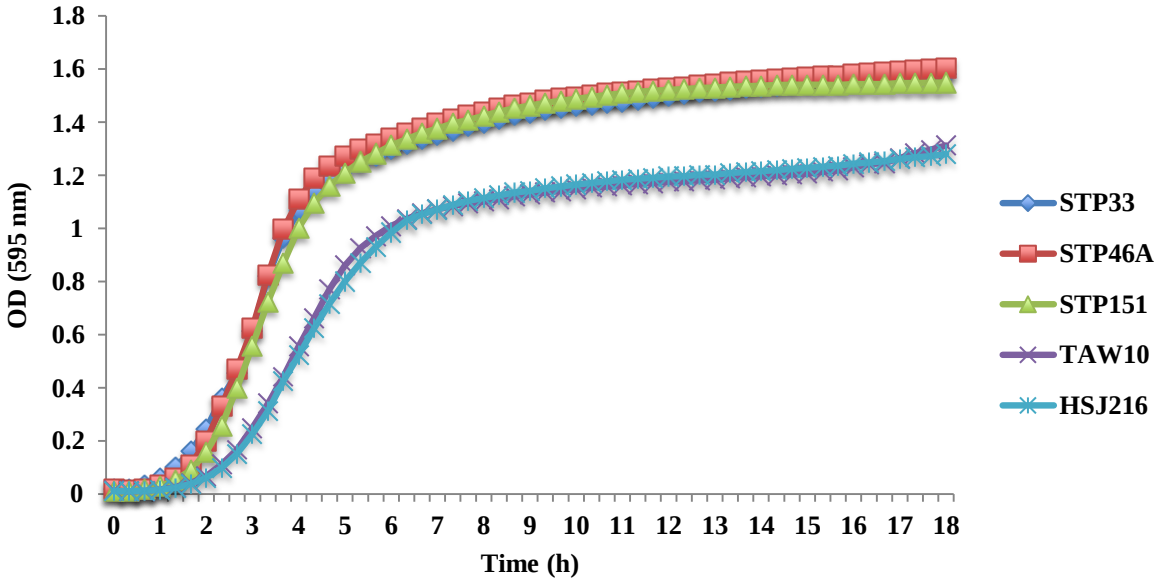

Figure 7 – Independent growth curves of selected MRSA strains resistant and hetero-resistant to SXT: STP33, STP46A, STP151, TAW10 and HSJ216.

In the co-culture experiments, the higher growth rates of STP33, STP46A and STP151 led to outcompete HSJ216 and TAW10. In fact, while all strains were initially inoculated in almost equal concentrations (Initial ratios: ST33/HSJ216 = 0.97; STP46A/HSJ216 = 1.15; ST151/HSJ216 = 1.33; STP151/TAW10 = 1.10), the African isolates represented 72 to 91% of the population after a 24 h incubation period (Final ratios: ST33/HSJ216 = 10.59; STP46A/HSJ216 = 10.51; ST151/HSJ216 = 2.58; STP151/TAW10 = 7.30). Interestingly, the final ratio was higher for the hetero-resistant isolates (STP33 and STP46A) compared with the full SXT-resistant STP151 isolate when co-cultured against HSJ216 – Figure 8 and Table 5.

Furthermore, the competition assay showed that the African strains exhibited a fitness advantage over HSJ216 and TAW10 isolates, with a relative competitive fitness varying from 1.07 to 1.30 – Table 5.

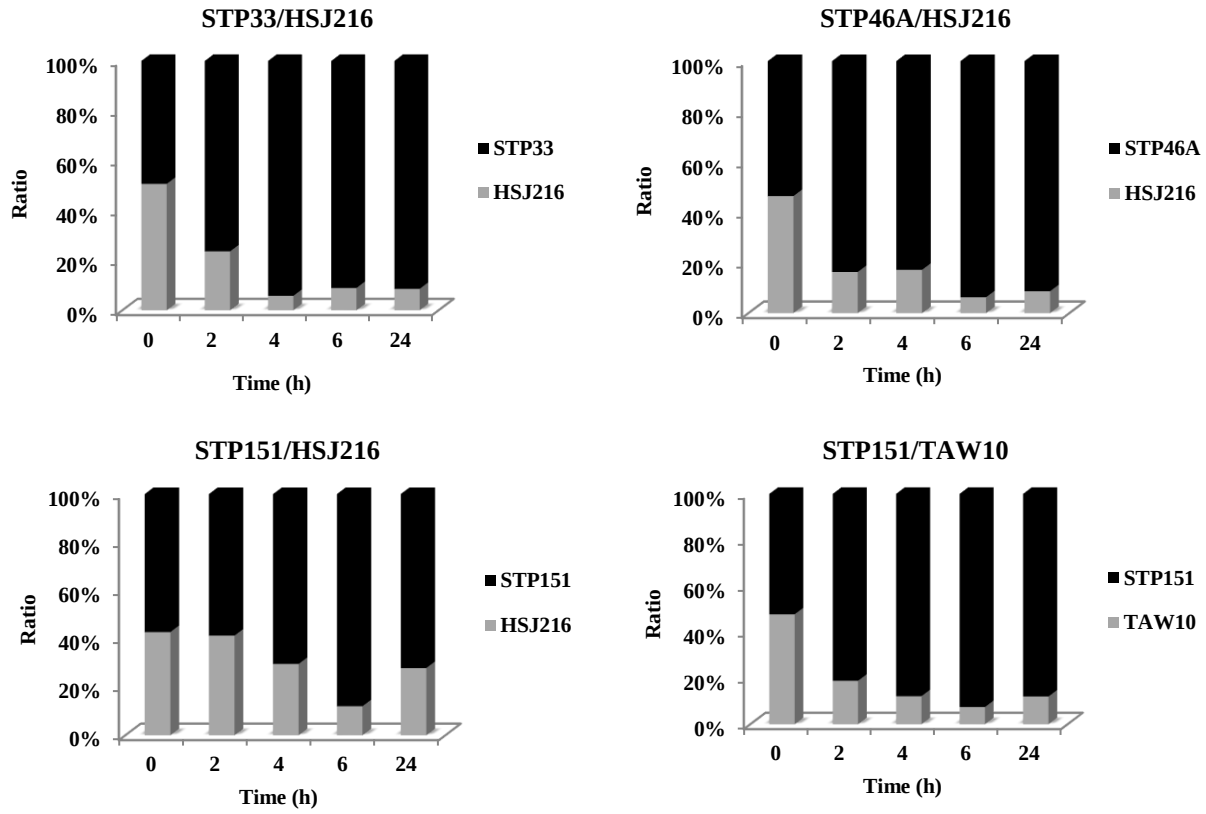

Figure 8 – Co-culture growth of the strain pairs STP33/HSJ216, STP46A/HSJ216, STP151/HSJ216 and STP151/TAW10.

Table 5 – Viable cell ratio and the relative fitness of each strain pairs.

Strain pairs	Viable cell ratio		Relative fitness, F
	Initial ratio (0 h)	Final ratio (24 h)	
STP33/HSJ216	0.97	10.59	1.30
STP46A/HSJ216	1.15	10.51	1.27
STP151/HSJ216	1.33	2.58	1.07
STP151/TAW10	1.10	7.30	1.21

Concerning the survival assay, a significantly increase in cfu count was observed for the African isolates when compared with TAW10 and HSJ216, after 6 h. Furthermore, after seven days, the two SXT hetero-resistant isolates (STP33 and STP46A) survived desiccation better, probably providing them an advantage in a model mimicking survival on hospital surface whereas the three full SXT-resistant isolates (STP151, TAW10 and HSJ216) presented

a lower survival rate, which might constitute a disadvantage regarding survival on dry surfaces. In addition, the daily death rate after day 1 seemed to be higher for STP151 and TAW10 – Figure 9 and Table 6.

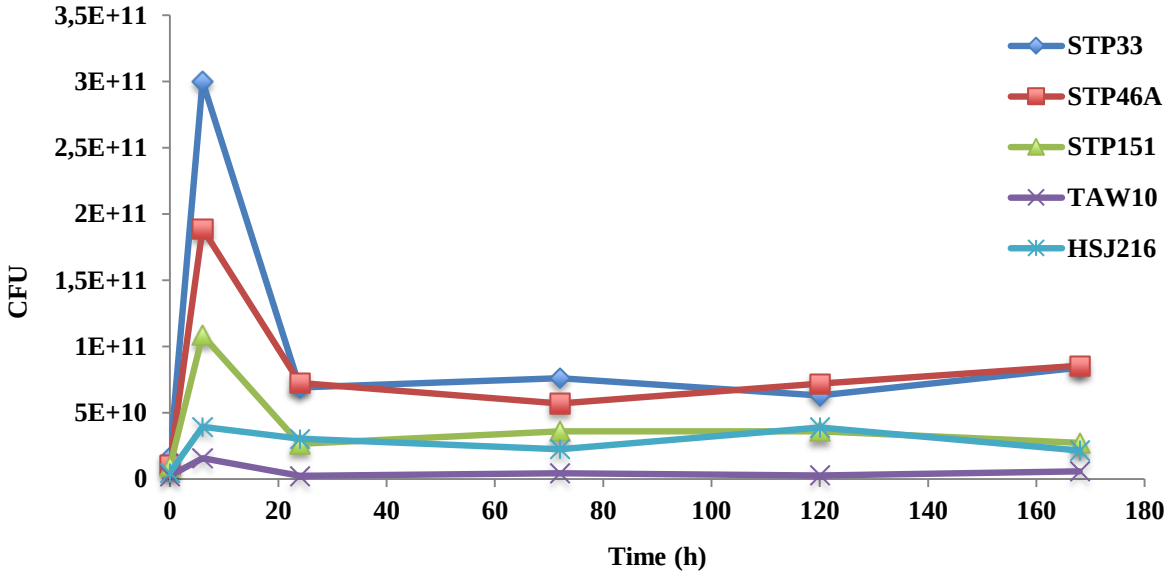

Figure 9 – Survival experiment.

Table 6 – Growth measurements from survival experiments.

Strain	Percentage of inoculum surviving to 6 h	Percentage of inoculum surviving to 7 days (168 h)	Daily death rate after day 1
STP33	1801.8	504.5	-1.42
STP46A	1766.4	799.1	-1.91
STP151	1112.8	280.5	-1.00
TAW10	648.8	237.6	-0.05
HSJ216	798.0	433.3	-1.81

2.2 Resistance to chemical stresses

The selected five isolates were tested under different stress conditions compared with their independent growth on TSB. A moderate to very high growth reduction was observed for all isolates in the presence of 4% (v/v) ethanol, in high saline (2.5 M NaCl) and in acidic (pH

4.5) conditions, respectively. In addition, the presence of 8.8 mM H_2O_2 in the medium completely inhibited the growth of all isolates – Figure 10 and Table 7.

Although no apparent difference in the growth was observed for the African isolates in alkaline conditions (pH 10), the percentage of growth of HSJ216 and TAW10 was significantly affected, being reduced to 62 and 72% respectively – Figure 10 and Table 7.

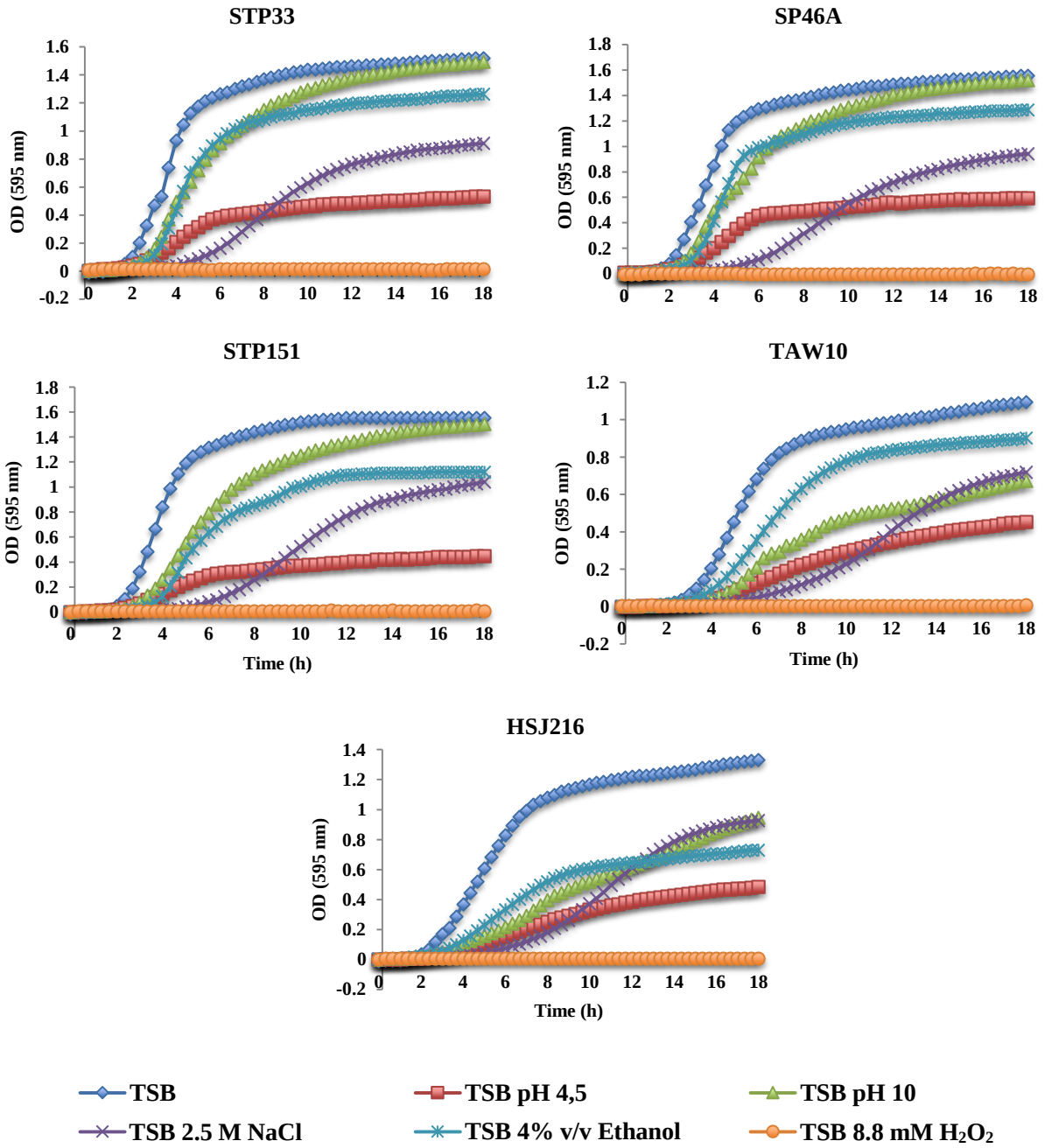

Figure 10 – Growth in the presence of several chemical stresses.

Table 7 – OD values after 24 h of growth in TSB and in the presence of several chemical stresses.

Strain	OD_{595} (%[1])					
	TSB[2]	TSB pH 4.5	TSB pH 10	TSB 2.5 M NaCl	TSB 4% v/v Ethanol	TSB 8.8 mM H_2O_2
STP33	1.52	0.54 (35.2)	1.50 (98.4)	0.92 (60.5)	1.27 (83.3)	0.01 (0.8)
STP46A	1.57	0.60 (38.2)	1.53 (97.5)	0.96 (61)	1.29 (82.6)	0 (0)
STP151	1.55	0.46 (29.5)	1.51 (97.1)	1.06 (68)	1.12 (72.3)	0.01 (0.6)
TAW10	1.11	0.46 (41.6)	0.69 (62.4)	0.73 (66)	0.90 (81.5)	0.01 (0.5)
HSJ216	1.34	0.49 (36.5)	0.97 (72.5)	0.94 (69.7)	0.74 (55)	0.01 (0.5)

[1] Percentage of growth under chemical stress regarding the own growth of each strain under control condition (TSB).
[2] Control condition.

The autolysis experiments demonstrated a slower rate of autolysis for TAW10 and HSJ216 isolates compared with isolates recovered from the African continent. For these, the autolysis activity was slightly higher for the full SXT-resistant STP151 isolate – Figure 11.

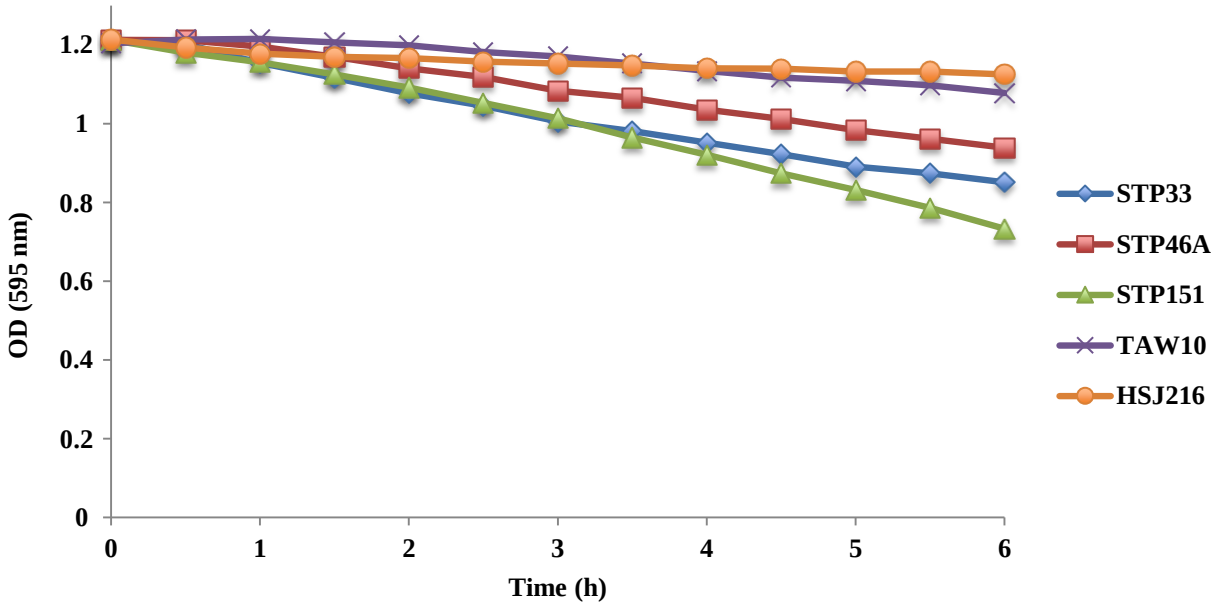

Figure 11 – Autolysis experiments.

CHAPTER IV – DISCUSSION AND CONCLUSIONS

The aim of the present study was to provide insights on the high rates of SXT-resistant *S. aureus* isolates in the African continent by firstly evaluating the prevalence of the different TMP resistance markers in São Tomé and Príncipe, Angola and Cape Verde. Unexpectedly, among our African collection, only isolates associated to one of the majors MRSA clones circulating in São Tomé and Príncipe (PFGE C-ST8-SCC*mec* $IV_c/IV_d/IV_g/V$) presented full resistance to SXT. The remaining isolates showed SXT hetero-resistance, regardless of their clonal types, associated to SMZ hetero-resistance or susceptible phenotypes. Antibiotic hetero-resistance phenotypes have been highly described among *S. aureus*, mainly associated with β-lactam and glycopeptide antibiotics (39, 45, 71, 102). However, to the best of our knowledge, this is the first study reporting SXT hetero-resistance. While some reports question the clinical significance of hetero-resistance (70, 92) others argue that there is deterioration in clinical outcomes due to hetero-resistance in *S. aureus* (14, 26). Lack of standard definition of hetero-resistance may lead to misidentification of homogeneous strains as hetero-resistant, hindering proper assessment of its clinical relevance. This phenotype has been relevant in recurrent infections, chronic infections, and infections with increasing mortality rates. Moreover, hetero-resistance has been misinterpreted when bacterial populations are only analysed by disk diffusion method, demonstrating the importance of determining MIC and implementing proper and standardized guidelines for practice concerning hetero-resistance detection (43).

Concerning the TMP resistance markers, all isolates from the African collection harboured *dfrG*, *dfrA* or both. *dfrG* gene was clearly the most common (81.1%) genetic determinant, found among both MRSA and MSSA isolates. Although *dfrG* gene was previously known to play minor importance among *S. aureus* isolates causing bacteraemia in humans (88), recent reports demonstrated the widespread prevalence of this gene among isolates recovered from sub-Saharan Africa and from travellers with SSTIs traveling from African to Europe, suggesting its potential origin in the African continent (85, 86). These findings contradict the previous prevailing concept that TMP resistance was mainly due to the presence of *dfrA* or to the F99Y mutation in the intrinsic *dfrB* gene (36, 65). Besides being present in several clonal lineages in the African continent, *dfrG* was also found in the two isolates belonging to the ST241 variant of the epidemic SXT-resistant Brazilian clone,

recovered from Taiwan. Although *drfG* was not detected among representatives of the original Brazilian clone (ST239-III) in our collection, this gene has been previously reported among sporadic isolates belonging to ST239-III from the Asiatic continent (104, 105). The fact that *dfrG* is usually located on small conjugative plasmids of approximately 500 bp, easily-transferable between different *S. aureus* genotypes (85), might explain its global prevalence on different clonal lineages namely in the African continent. In the present study, *dfrA* gene was mainly associated to the community-associated West Australia MRSA-2 clone (ST88-IV), which is a major clonal type currently circulating in Africa (1, 33). This observation is in agreement with previous studies reporting that *dfrA* is mainly associated with large plasmids of the psK1 family that have the tendency to be clonal, suggesting its inclusion in a local clone (52, 85). Interestingly, only one isolate among representatives of the Brazilian clone harboured *dfrA*. This isolate, was recovered from Portugal and belongs to a separate clade among ST239 lineage (58), which may explain the presence of this resistance determinant. Only four MRSA strains from the African collection carried both *dfrG* and *dfrA* genes. This phenomenon of carrying both TMP genetic determinants has already been described to be rare among *S. aureus* isolates in the study conducted by Nurjadi *et al.* (85).

The *dfrK* gene was not detected in our study, neither in isolates recovered from the African collection, or in representatives of the Brazilian clone. This gene is commonly located in the 40-kb plasmid pKKS2187 and has been mainly found in livestock-associated *S. aureus* multilocus sequence type ST398 from Europe (10, 35, 47, 67). To date, *dfrK* gene was detected outside Europe in only one MSSA isolate causing SSTIs in a subject in Gabon (85).

Among the 15 representatives of the Brazilian clone that did not harbour *dfrG*, *dfrA* or *dfrK*, only 10 isolates amplified the intrinsic *dfrB* gene that was further sequenced. The sequencing results reveal the common F99Y mutation among all our isolates, consistently associated with TMP resistance. In addition, the R150H mutation, also related with TMP resistance but not so frequently described, was also detected in all 10 isolates. In concordance with previous studies (36, 85), we observed that *dfrB* mutation confers intermediate-level TMP resistance (up to 256 μg/mL) in *S. aureus*, when compared with isolates that carried *dfrG* and/or *dfrA* as TMP genetic determinant that presented high-level resistance. Further investigation is needed concerning the five remaining isolates for which we did not find any of the *dfr* genes described so far.

The wide prevalence of *dfrG* among *S. aureus* is of major concern for the development of new antifolate antimicrobials (20, 48). Usually, drug testing of new potential antifolate antimicrobials chemicals *in vitro* are performed against isolates carrying *dfrB* mutations or *dfrA* gene (48). Since there is evidence that *dfrG* is widely disseminated in Africa, further antimicrobial testing of new antifolate compounds must include *S. aureus* expressing *dfrG*-encoded DHFR.

Three main MRSA lineages are currently circulating in Portuguese-speaking African countries, i.e. A-ST5-IV_a, B-ST88-IV_a, and C-ST8- IV_c/IV_d/IV_g/V clones, all SXT resistant (clone C- ST8- IV_c/IV_d/IV_g/V) or SXT hetero-resistant (clones A--ST5-IV_a and B-ST88-IV_a) (33). However, factors contributing to the epidemic success of these clonal types are still unclear, namely their advantage compared to the SXT-resistant Brazilian clone (ST239-III), which is widely spread all over the word and prevalent in some other countries in Africa but not detected in the three Portuguese-speaking African countries.

Thouverez *et al*. (117) demonstrated that fitness advantages are crucial in the dissemination of MRSA clones in hospitals, where there is a strong selective antibiotic pressure. Our results demonstrated that the prevalent clonal types in Africa were fittest, both in independent and co-culture growth, when tested against representatives of the Brazilian clone (ST239) and its variant ST241. Interestingly, the Brazilian clone replaced previously well-established MRSA in several countries (3, 27, 32, 128) and therefore is likely to have an advantage. Even knowing that antibiotic resistance may contribute to a decreased fitness of bacteria (83), in our study the full SXT resistance of ST151 isolate did not confer any fitness cost regarding the hetero-resistant counterparts, and outcompeted similarly the Brazilian clone. This observation may be explained by the fact that isolates hetero-resistant to SXT harbour the same genetic determinants than the full resistant isolates and therefore there is no additional cost.

S. aureus is extremely tolerant to desiccation and therefore can survive in hostile environments outside hosts (29). In the present study, isolates belonging to the three major clones circulating in Portuguese-speaking African countries and also the two representatives of the Brazilian clone and variant could survive for several days after drying on plastic, which is consistent with previous results obtained by Chaibenjawong and Foster (23). However, the African isolates survived better to desiccation conditions, which could explain their clonal

persistence and dissemination in healthcare settings, with consequent transmission to patients. Hence, the longer survival of these microorganisms on dry surfaces, underscores the importance of meticulous contact control procedures and through disinfection of hospital surfaces, to minimize the spread of multi-resistant microorganism, such as MRSA.

To colonize, survive and cause infection, *S. aureus* has been developing resistance to a wide range of environment- and host-related stresses (29). In the present study, we found that isolates belonging to successful African clones as well as representatives of the pandemic MRSA ST239 could survive at approximately 60% of their control growth when exposed to high salinity conditions. High NaCl concentration promotes the accumulation of cytoplasmatic osmoprotectants and shortens the interpeptide bridges of cell-wall peptidoglycans to confer mechanical strength on the cell to resist drastic changes in osmotic pressure (119).

Oxidative stress is one of the mechanisms adopted by phagocytic cells to kill bacteria by producing reactive oxygen species (ROS), that are known to cause lethal damage to DNA, lipids and proteins of the bacteria. The most frequent oxidative stress resistance in *S. aureus* is mediated by the production of detoxification enzymes, such as superoxide dismutase (SOD), that will neutralize ROS (51). In a previous study, the European widely disseminated EMRSA-15, was much more resistant to oxidative stress than the displaced predominant Italian clone (ST228) (11). However, in our study, oxidative conditions completely inhibited the growth in all isolates and therefore cannot explain their increased capacity of dissemination.

S. aureus must successfully adapt to transient pH variations due to natural or artificial circumstances (11). Although no apparent difference was observed concerning growth rates of the African isolates compared to ST239/ST241 MRSA isolates under acidic pH, it was not the case under alkaline conditions, where African isolates were considerably less affected, which can contribute to the success of these lineages.

Autolysis is essential in cell wall turnover, and can usually be activated by adverse physiological conditions (54). Isolates belonging to the Brazilian MRSA clone presented a lower autolytic activity than *S. aureus* isolates from African clones and therefore better survived the challenge attack on cell wall stability posed by Triton X-100. It has been suggested that developmental of cell death confers an intrinsic advantage when bacteria are exposed to diverse stress, including antibiotic exposure, by generating a diverse but stable dispersed population (95). In addition, the two SXT hetero-resistant isolates, STP33 and STP46A,

showed a reduced autolysis rate compared with the full SXT-resistant STP151, so we can speculate that full resistance to SMZ and SXT has a negative effect on the cell stability.

While we have not exhausted all growth conditions encountered by *S. aureus* in the hospital and healthcare environments, the fact that all three isolates recovered from São Tomé and Príncipe were dominant in the great majority of the assays, including suboptimal survival conditions, suggests that it might be a significant fitness advantage.

In summary, we reported for the first time SXT-hetero-resistance, a phenomenon that needs further exploitation since it is highly frequent in *S. aureus* isolates from Africa. We also provided the first data on the prevalence of the TMP resistance genes in *S. aureus* isolates recovered from Portuguese-speaking African countries and evidenced a wide prevalence of *dfrG* gene, reinforcing its potential origin in the African continent. Since these countries present important demographic and economic exchanges with Portugal, and *drfG* is transferable in a mobile genetic element, future spread of *dfrG* within MRSA in populations where antifolate resistance is currently considered to be low should be monitored. Moreover, we showed that the epidemiological success of the three major MRSA clones in Portuguese-speaking African countries may be due (i) to their capacity to survive under different stress situations including a better adaptation to alkaline conditions, and (ii) to their potential to outgrow other SXT-resistant MRSA clones such as the Brazilian ST239-III, a pandemic MRSA clone present in other African countries.

CHAPTER V – REFERENCES

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CHAPTER VI – ANNEXES

Annex 1

Supplementary table 1S – Trimethoprim, sulfonamide and trimethoprim-sulfamethoxazole resistance in *S. aureus*.

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]					TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
ANG17A	Angola	MR	A	5	**R**	> 1024	**HR**	2048	**HR**		+	-			
ANG19	Angola	MR	A	5	**R**	1024	**HR**	1024	**HR**		+	-			
ANG112	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG116	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG163	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG217	Angola	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			
ANG231A	Angola	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			
ANG243	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG245	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG284	Angola	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			
ANG285	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG306	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG313A	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG316A	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG364	Angola	MR	A	5	**R**	> 1024	**S**	128	**HR**		+	-			
ANG451	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG474	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG516A	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG525A	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG561A	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG567	Angola	MR	A	5	**R**	> 1024	**S**	256	**HR**		+	-			
ANG570A	Angola	MR	A	5	**R**	> 1024	**S**	128	**HR**		+	-			
ANG631	Angola	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]						TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
ANG636A	Angola	MR	A	5	**R**	> 1024	**HR**	2048	**HR**		+	-			
ANG654	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG661	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG786	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG788	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG792	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG83A	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG898	Angola	MR	A	5	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG912	Angola	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			
ANG915	Angola	MR	A	5	**R**	> 1024	**S**	128	**HR**		+	-			
ANG916	Angola	MR	A	5	**R**	> 1024	**S**	128	**HR**		+	-			
STP46A	STP	MR	A	5	**R**	> 1024	**HR**	512	**HR**		+	-			
STP497	STP	MR	A	5	**R**	1024	**HR**	1024	**HR**		+	-			
CV492	Cape Verde	MR	ABB	5	**R**	1024	**HR**	1024	**HR**		-	+			
CV495	Cape Verde	MR	ABB	5	**R**	1024	**HR**	2048	**HR**		-	+			
ANG634	Angola	MR	AQ	8	**R**	1024	**S**	< 64	**HR**		+	-			
ANG874	Angola	MR	AQ	8	**R**	> 1024	**S**	256	**HR**		+	+			
ANG880	Angola	MR	AQ	8	**R**	1024	**S**	256	**HR**		+	+			
ANG49	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG137	Angola	MR	B	88	**R**	> 1024	**HR**	2048	**HR**		-	+			
ANG216	Angola	MR	B	88	**R**	> 1024	**HR**	2048	**HR**		-	+			
ANG270A	Angola	MR	B	88	**R**	1024	**HR**	1024	**HR**		-	+			
ANG278A	Angola	MR	B	88	**R**	1024	**HR**	1024	**HR**		+	+			
ANG293A	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG365	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG612A	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		+	+			
ANG793	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]						TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
ANG842	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG895	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG900A	Angola	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
CV188	Cape Verde	MR	B	88	**R**	1024	**HR**	512	**HR**		-	+			
CV346	Cape Verde	MR	B	88	**R**	1024	**HR**	1024	**HR**		-	+			
STP33	STP	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
STP74A	STP	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
STP254	STP	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
STP363	STP	MR	B	88	**R**	1024	**HR**	1024	**HR**		-	+			
STP377A	STP	MR	B	88	**R**	1024	**HR**	1024	**HR**		-	+			
STP412	STP	MR	B	88	**R**	> 1024	**HR**	1024	**HR**		-	+			
ANG40	Angola	MR	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
ANG202	Angola	MR	C	8	**R**	> 1024	**R**	1024	**R**	8	-	+			
ANG268	Angola	MR	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
ANG304	Angola	MS	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
ANG358	Angola	MR	C	8	**R**	1024	**R**	1024	**R**	>32	-	+			
ANG573A	Angola	MR	C	8	**R**	> 1024	**R**	1024	**R**	>32	+	-			
ANG725	Angola	MR	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
ANG743	Angola	MR	C	8	**R**	> 1024	**R**	1024	**R**	16	-	+			
STP59	STP	MR	C	8	**R**	1024	**R**	1024	**R**	6	+	-			
STP151	STP	MR	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
STP280	STP	MR	C	8	**R**	> 1024	**R**	2048	**R**	12	+	-			
STP292	STP	MR	C	8	**R**	> 1024	**R**	2048	**R**	16	+	-			
STP297	STP	MR	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
STP34A	STP	MS	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
STP350A	STP	MR	C	8	**R**	256	**R**	2048	**R**	16	+	-			
STP353	STP	MR	C	8	**R**	1024	**R**	2048	**R**	6	+	-			

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]						TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
STP368A	STP	MR	C	8	**R**	1024	**R**	2048	**R**	16	+	-			
STP389A	STP	MS	C	8	**R**	> 1024	**R**	2048	**R**	>32	+	-			
STP392	STP	MR	C	8	**R**	1024	**R**	2048	**R**	16	+	-			
STP465A	STP	MS	C	8	**R**	> 1024	**R**	1024	**R**	>32	+	-			
ANG25	Angola	MR	D	72	**R**	1024	**S**	256	**HR**		+	-			
ANG161	Angola	MR	D	72	**R**	> 1024	**S**	256	**HR**		+	-			
ANG503	Angola	MR	D	72	**R**	1024	**HR**	512	**HR**		+	-			
ANG784	Angola	MR	D	72	**R**	> 1024	**S**	64	**HR**		+	-			
ANG477	Angola	MR	J	789	**R**	1024	**HR**	1024	**HR**		+	-			
ANG328A	Angola	MS	K	15	**R**	1024	**S**	256	**HR**		+	-			
ANG545	Angola	MS	K	15	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG565	Angola	MS	K	15	**R**	> 1024	**HR**	1024	**HR**		+	-			
ANG765	Angola	MS	K	15	**R**	> 1024	**S**	1024	**HR**		+	-			
ANG798	Angola	MS	K	15	**R**	> 1024	**HR**	1024	**HR**		+	-			
CV234	Cape Verde	MS	K	15	**R**	> 1024	**HR**	512	**HR**		+	-			
CV261	Cape Verde	MS	K	15	**R**	> 1024	**HR**	2048	**HR**		+	-			
CV327A	Cape Verde	MS	K	15	**R**	> 1024	**HR**	512	**HR**		+	-			
CV561	Cape Verde	MS	K	15	**R**	> 1024	**S**	< 64	**HR**		+	-			
CV562A	Cape Verde	MS	K	15	**R**	> 1024	**S**	256	**HR**		+	-			
CV564	Cape Verde	MS	K	15	**R**	> 1024	**HR**	512	**HR**		+	-			
CV669A	Cape Verde	MS	K	15	**R**	> 1024	**S**	256	**HR**		+	-			
STP126	STP	MS	K	15	**R**	> 1024	**S**	128	**HR**		+	-			
STP252A	STP	MS	K	15	**R**	> 1024	**S**	< 64	**HR**		+	-			
STP80	STP	MS	K	15	**R**	> 1024	**S**	256	**HR**		+	-			
STP286	STP	MS	K	15	**R**	> 1024	**HR**	512	**HR**		+	-			
STP381	STP	MS	K	15	**R**	> 1024	**S**	128	**HR**		+	-			
STP411	STP	MS	K	15	**R**	> 1024	**HR**	1024	**HR**		+	-			

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]						TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
STP428A	STP	MS	K	15	R	> 1024	S	128	HR		+	-			
STP463	STP	MS	K	15	R	> 1024	S	128	HR		+	-			
STP479.1	STP	MS	K	15	R	> 1024	HR	512	HR		+	-			
ANG461	Angola	MS	M	152	R	1024	S	256	HR		+	-			
STP327	STP	MS	M	152	R	1024	S	128	HR		+	-			
ANG428	Angola	MS	N	8	R	> 1024	S	128	HR		+	-			
ANG510A	Angola	MS	N	8	R	> 1024	S	128	HR		+	-			
ANG432	Angola	MS	O	121	R	1024	S	256	HR		+	-			
STP196	STP	MS	O	121	R	1024	HR	512	HR		+	-			
STP214	STP	MS	O	121	R	> 1024	S	128	HR		+	-			
STP351	STP	MS	O	121	R	> 1024	S	< 64	HR		+	-			
STP376	STP	MS	O	121	R	> 1024	S	< 64	HR		+	-			
ANG407	Angola	MR	U	5	R	> 1024	S	< 64	HR		+	-			
ANG416	Angola	MR	U	2629	R	> 1024	HR	512	HR		+	-			
ANG36	Angola	MS	W	25	R	> 1024	S	128	HR		+	-			
ANG286	Angola	MS	X	30	R	> 1024	HR	512	HR		+	-			
ANG291A	Angola	MS	X	30	R	> 1024	S	256	HR		+	-			
ANG899	Angola	MR	X	30	R	> 1024	S	256	HR		+	-			
ARG106	Argentina	MR	NA	239	R	256	R	2048	R	>32	-	-	-	-	
ARG266	Argentina	MR	NA	239	R	256	R	2048	R	>32	-	-	-	+	F99Y; R150H
BZ17	Brazil	MR	NA	239	R	256	R	2048	R	>32	-	-	-	+	F99Y; R150H
BZ20	Brazil	MR	NA	239	R	256	R	1024	R	24-32	-	-	-	-	
BZ23	Brazil	MR	NA	239	R	256	R	2048	R	24-32	-	-	-	-	
CHL1	Chile	MR	NA	239	R	256	R	1024	R	>32	-	-	-	+	F99Y; R150H
CHL10	Chile	MR	NA	239	R	256	R	1024	R	>32	-	-	-	+	F99Y; R150H
CHL33	Chile	MR	NA	239	R	256	R	1024	R	>32	-	-	-	+	F99Y; R150H

Strain	Origin[1]	MR/ MS[2]	PFGE[3]	ST[4]	Phenotypic antifolate resistance[5]						TMP resistance gene				
					TMP[6]	TMP (µg/mL)	SULF[6]	SMZ (µg/mL)	SXT	SXT (µg/mL)	*dfrG*	*dfrA*	*dfrK*	*dfrB*	DfrB aa substitution
TAW10	Taiwan	MR	NA	241	**R**	1024	**R**	1024	**R**	> 32	+	-			
TAW101	Taiwan	MR	NA	241	**R**	> 1024	**R**	1024	**R**	>32	+	-			
TAW111	Taiwan	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	-	
URU1	Uruguay	MR	NA	239	**R**	256	**R**	2048	**R**	>32	-	-	-	+	F99Y; R150H
URU11	Uruguay	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	+	F99Y; R150H
URU14	Uruguay	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	+	F99Y; R150H
FFP126	Portugal	MR	NA	239	**R**	256	**R**	2048	**R**	>32	-	+			
FFP200	Portugal	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	+	F99Y; R150H
HSJ216	Portugal	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	-	
IPO118	Portugal	MR	NA	239	**R**	256	**R**	1024	**R**	>32	-	-	-	+	F99Y; R150H

[1] STP, São Tomé and Príncipe.
[2] MR, Methicillin-resistant; MS, Methicillin-susceptible.
[3] PFGE, Pulsed-filed gel electrophoresis; NA, Not applied.
[4] ST, Sequence type.
[5] TMP, Trimethoprim; SULF, Sulfonamide; SMZ; Sulfamethoxazole; SXT, Trimethoprim/sulfamethoxazole; R, Resistant; HR, Hetero-resistant; S, Susceptible. Antimicrobial phenotypes for TMP and SMZ were determining by interpreting the results of inhibition zone of the disk diffusion method and the microdilution assay using Tecan apparatus. Antimicrobial phenotypes for SXT were determined by interpreting the results of inhibition zone of the disk diffusion method and/or E-test strips.